09806555 .070299

JC03 Rec'd PCT/PTO 30 MAR 2001

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Date of Deposit March 30, 2001 Label Number: EL834597709US

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Guy Beardsley
Printed name of person mailing correspondence

Guy Beardsley
Signature of person mailing correspondence

Substitute Form PTO 1390 U.S. Department of Commerce Patent and Trademark Office

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

Attorney's Docket Number: 50125/022001

U.S. Application Number: 09/806555

| INTERNATIONAL APPLICATION NUMBER | INTERNATIONAL FILING DATE | PRIORITY DATE CLAIMED |
|---|---|---|
| PCT/EP99/07296 | 01.10.99 | 02.10.98 |

| | |
|---|---|
| TITLE OF INVENTION: | TISSUE-BINDING PEPTIDES, THEIR IDENTIFICATION, PREPARATION AND USE |
| APPLICANTS FOR DO/EO/US: | Jürgen Schrader et al. |

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

| | |
|---|---|
| 1. | ■ This is a FIRST submission of items concerning a filing under 35 U.S.C. § 371. |
| 2. | □ This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. § 371. |
| 3. | ■ This is an express request to begin national examination procedures (35 U.S.C. § 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. § 371(b) and PCT Articles 22 and 39(1). |
| 4. | ■ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date. |
| 5. | A copy of the International Application as filed (35 U.S.C. § 371(c)(2)).<br>□ a. is transmitted herewith (required only if not transmitted by the International Bureau).<br>■ b. has been transmitted by the International Bureau.<br>□ c. Is not required, as the application was filed with the United States Receiving Office (RO/US). |
| 6. | ■ A translation of the International Application into English (35 U.S.C. § 371(c)(2). |
| 7. | Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. § 371(c)(3)).<br>□ a. are transmitted herewith (required only if not transmitted by the International Bureau).<br>■ b. have been transmitted by the International Bureau.<br>□ c. have not been made; however, the time limit for making such amendments has NOT expired.<br>□ d. have not been made and will not be made. |
| 8. | ■ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. § 371(c)(3)). |
| 9. | ■ An oath or declaration of the inventors (35 U.S.C. § 371(c)(4)). (Unsigned) |
| 10. | □ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371 (c)(5). |
| 11. | □ An Information Disclosure Statement under 37 C.F.R. §§ 1.97 and 1.98. |
| 12. | □ An assignment for recording. A separate cover sheet in compliance with 37 §§ 3.28 and 3.31 is included. |
| 13. | ■ A FIRST preliminary amendment.<br>□ A SECOND or SUBSEQUENT preliminary amendment. |
| 14. | □ A substitute specification. |
| 15. | □ A change of power of attorney and/or address letter. |
| 16. | ■ Other items or information: PCT/IPEA/409 form and PCT/ISA/210 form |

09/806555

JC08 Rec'd PCT/PTO 30 MAR 2001

| | | | | |
|---|---|---|---|---|
| 17. | ■ The following fees are submitted: | | | |
| | BASIC NATIONAL FEE (37 C.F.R. § 1.492(A)(1)-(5)): | | | |
| | Neither international preliminary examination fee (37 C.F.R. § 1.482) nor international serach fee (37 C.F.R. § 1.455(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO | $ 1000.00 | | |
| | International preliminary examination fee (37 C.F.R. § 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO | $ 860.00 | $860.00 | |
| | International preliminary examination fee (37 C.F.R. § 1.482) not paid to USPTO but international search fee (37 C.F.R. § 1.445(a)(2)) paid to USPTO | $710.00 | | |
| | International preliminary examination fee (37 C.F.R. § 1.482) paid to USPTO but all claims did not satisfy provisions of PCT Article 33(1) - (4) | $ 690.00 | | |
| | International preliminary examination fee paid to USPTO (37 C.F.R. § 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4) | $ 100.00 | | |

| | | | | | |
|---|---|---|---|---|---|
| ENTER APPROPRIATE BASIC FEE AMOUNT = | | | | $860.00 | |
| Surcharge of $130 for furnishing the oath or declaration later than □ 20 OR □ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(e)). | | | | $ | |
| CLAIMS | NUMBER FILED | NUMBER EXTRA | RATE | | |
| Total claims | 30 - 20 = | 10 | x $18 | $ 180.00 | |
| Independent claims | 2 - 3 = | 0 | x $80 | $ | |
| Multiple dependent claims (if applicable) | | | + $270 | $ 270.00 | |
| TOTAL OF ABOVE CALCULATIONS = | | | | $ 1310.00 | |
| **SMALL ENTITY STATUS:** | | | | | |
| Applicant claims small entity status under 37 CFR 1.27. | | | | | |
| Reduction of 1/2 for filing by small entity, if applicable Applicant claims small entity status under 37 C.F.R. § 1.27 | | | | $655.00 | |
| SUBTOTAL = | | | | $ 655.00 | |
| Processing fee of $130.00 for furnishing the English translation later than □ 20 OR □ 30 months from the earliest claimed priority date (37 C.F.R. § 1.492(f)). + | | | | $ | |
| TOTAL NATIONAL FEE = | | | | $ 655.00 | |
| Fee for recording the enclosed assignment (37 C.F.R. 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 C.F.R. §§ 3.28, 3.31). $40.00 per property. + | | | | $ | |
| TOTAL FEES ENCLOSED = | | | | $ 655.00 | |
| | | | | Amount to be refunded | $ |
| | | | | charged | $ |

■ a. A check in the amount of $655.00 to cover the above fees is enclosed.
□ b. Please charge my Deposit Account No. 03-2095 in the amount of $ [**.**] to cover the above fees.
■ c. The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment, to Deposit Account No. 03-2095.

NOTE: Where an appropriate time limit under 37 C.F.R. §§ 1.494 or 1.495 has not been met, a petition to revive (37 C.F.R. § 1.137(a) or (b) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

Karen L. Elbing, Ph.D.
Clark & Elbing LLP
176 Federal Street
Boston, MA 02110-2214
Telephone: 617-428-0200
Facsimile: 617-428-7045

Signature
Karen L. Elbing, Ph.D.
Reg. No. 35,238

PATENT
ATTORNEY DOCKET NO. 50125/022001

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Date of Deposit March 30, 2001 Label Number: EL834597709US

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Guy Beardsley
Printed name of person mailing correspondence

Guy Beardsley
Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| Applicant: | Jürgen Schrader et al. | Art Unit: | |
| Serial No.: | Not yet assigned | Examiner: | |
| Filed: | March 30, 2001 | Customer No.: | 21559 |
| Title: | TISSUE-BINDING PEPTIDES, THEIR IDENTIFICATION, PREPARATION AND USE | | |

Assistant Commissioner For Patents
Washington, DC 20231

PRELIMINARY AMENDMENT

Prior to examination, kindly amend the above-referenced application as follows.

In the claims:

Replace current claims 1-17 with the following new claims 18-39.

18. A tissue-binding peptide selected from the group consisting of peptides having the amino acid sequence GEGRTVVLSF, AWCRGGILGDAM, GNLVDLVVGFDD, RVSPPKKSGGGV, GSSKWGLTXKCG, RGGVRQRSRGRR, GEGRTVVCRS, and SQRWTALWQWIG, and variants thereof.

19. A nucleic acid coding for a tissue-binding peptide as claimed in claim 18.

20. A nucleic acid as claimed in claim 19 selected from the group consisting of nucleic acids having the nucleotide sequence
GGCGAGGGGCGAACAGTCGTATTGTCGTTCG,
GCCTGGTGTCGGGGGGGTATCCTGGGCGACGCTATG,

GGAAACCTGGTGGATCTAGTTGTGGGTTTTGACGAC,

CGGGTGAGTCCGCCAAAGAAGTCGGGGGGCGGCGTG,

GGGAGTAGCAAGTGGGGATTGACTTAAAAATGTGGG,

CGCGGGGGAGTCCGCCAAAGAAGTCGGGGGCGGCGT,

GGCGAGGGGCGAACAGTCGTATGTCGTTCG, and

TCCCAGAGGTGGACTGCACTCTGGCAATGGATCGGG,

and variants thereof.

21. A vector comprising a nucleic acid as claimed in claim 19.

22. A method for preparing a tissue-binding peptide as claimed in claim 18, characterized in that the peptide is prepared either by chemical synthesis or by genetic manipulation.

23. A method for finding a tissue-binding peptide where the peptide or peptides are present in a peptide-presenting bacterial library, the method comprising the following steps:

(a) bringing a tissue into contact with one or more peptides, and

(b) isolating one or more tissue-binding peptides.

24. The method as claimed in claim 23, characterized in that the peptides isolated in step (b) are brought into contact repeatedly one or more times with the same or with a different tissue.

25. The method as claimed in claim 23, characterized in that the tissue is a diseased tissue.

26. The use of a tissue-binding peptide as claimed in claim 18 for the tissue-specific transfer of substances.

27. The use of tissue binding peptide obtained by the method as claimed in claim 23 for the tissue-specific transfer of substances.

28. The use as claimed in claim 26 or 27, characterized in that the gene transfer proceeds virally and/or nonvirally.

29. The use as claimed in claim 26 or 27 characterized in that the gene transfer proceeds with the aid of liposomes.

30. The use as claimed in claim 26 or 27, characterized in that one or more tissue-binding peptides are bound via a positively charged domain to one or more nucleic acids.

31. The use of a tissue-binding peptide as claimed in claim 18 for altering the tropism of viruses.

32. The use of a tissue-binding peptide obtained by the method as claimed in claim 23 for altering the tropism of viruses.

33. The use of a tissue-binding peptide as claimed in claim 18 for providing a diagnostic aid for visualizing pathologically altered tissue and/or various sections of the vascular system.

34. The use of a tissue-binding peptide obtained by the method as claimed in claim 23 for providing a diagnostic aid for visualizing pathologically altered tissue and/or various sections of the vascular system.

35. The use as claimed in claim 33, characterized in that the pathologically altered tissue is selected from the group consisting of a pathologically altered vessel, in cases of inflammation, arteriosclerosis, vessels which supply tumor tissue, and tissue with proliferating smooth muscle cells of the vascular system.

36. The use as claimed in claim 34, characterized in that the pathologically altered tissue is selected from the group consisting of a pathologically altered vessel, in cases of inflammation, arteriosclerosis, vessels which supply tumor tissue, and tissue with proliferating smooth muscle cells of the vascular system.

37. The use as claimed in claim 33 or 34, characterized in that the tissue-binding peptide is labeled.

38. A pharmaceutical and/or diagnostic aid comprising one or more tissue-binding peptides as claimed in claim 18, one or more nucleic acids as claimed in claim 19, or one or more tissue-binding peptides obtained by the method as claimed in claim 23, and, where appropriate, suitable excipients and/or additives.

39. A composition comprising one or more tissue-binding peptides as claimed in claim 18, one or more nucleic acids as claimed in claim 19, or one or more tissue-binding peptides obtained by the method as claimed in claim 23, and another substance, and, where appropriate, suitable excipients and/or additives.

If there are any other charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 30 March 2001

Karen L. Elbing, Ph.D.
Reg. No. 35,238

Clark & Elbing LLP
176 Federal Street
Boston, MA 02110-2214
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**PATENT**
**ATTORNEY DOCKET NUMBER: 50125/022001**

Certificate of Mailing: Date of Deposit: June 28, 2001

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Colleen Coyne — Printed name of person mailing correspondence

Colleen Coyne — Signature of person mailing correspondence

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

| | | | |
|---|---|---|---|
| Applicant: | Jürgen Schrader et al. | Art Unit: | |
| Serial No.: | 09/806,555 | Examiner: | |
| Filed: | March 30, 3001 | Customer No.: | 21559 |
| Title: | TISSUE-BINDING PEPTIDES, THEIR IDENTIFICATION, PREPARATION AND USE | | |

Assistant Commissioner for Patents
Washington, D.C. 20231

PRELIMINARY AMENDMENT

Prior to substantive examination, kindly amend the above-referenced application as follows.

In the Specification:

Insert the enclosed Sequence Listing at the end of the Application.

At page 5, line 20, after "sequence" insert --(SEQ ID NOS: 1-8)--.

At page 6, line 7, after "GWYG" insert --(SEQ ID NO: 9)--.

At page 8, line 8, after "sequence:" insert --(SEQ ID NOS: 10-17)--.

At page 20, line 6, after "DNA sequences" insert --(SEQ ID NOS: 10-17)--.

At page 20, line 6, after "peptide sequences" insert --(SEQ ID NOS: 1-8)--.

If there are any charges, or any credits, please apply them to Deposit Account No. 03-2095.

Respectfully submitted,

Date: 28 June 2001

Karen L. Elbing, Ph.D.
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\\Clark-w2k1\documents\50125\50125.022001 Preliminary Amendment for Sequence Listing.wpd

SEQUENCE LISTING

<110> Schrader, Jurgen
Herrmann, Andreas

<120> Tissue-Binding Peptides, Their Identification, Preparation and Use

<130> 50125/022001

<140> US 09/806,555
<141> 2001-03-30

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# Tissue-binding peptides, their identification, preparation and use

The present invention relates to tissue-binding peptides, their identification with the aid of tissues, their preparation and use as pharmaceutical or diagnostic aid.

The pathogenesis of arteriosclerosis is increasingly attributed to damage to the endothelium resulting in endothelial dysfunction. Endothelial dysfunction is reflected in particular by an altered pattern of expression of surface proteins (selectins, adhesion molecules) but also by altered rates of synthesis of growth hormones. Thus, for example, in the early stage of arteriosclerosis, increased expression of E- and P-selectin and of the cellular adhesion molecules VCAM and ICAM leads to migration of monocytes into the vessel wall. Conversion of the monocytes into macrophages leads to an altered synthesis of growth factors, which stimulate in particular smooth muscle cells to proliferate and release mediators having inflammatory effects.

Drug therapy of arteriosclerosis attempts to diminish the causes at an early time, where possible before manifestation. A main aim in this connection is to reduce the elevated plasma cholesterol level by increasing the excretion of cholesterol metabolites (bile acids) or by decreasing cholesterol biosynthesis. Because of the counter-regulation initiated by the body it is possible to reduce the total cholesterol level by only about 30% with a drug therapy. Since this method is moreover unsuitable for inducing regression of existing arteriosclerosis, percutaneous transluminal angioplasty (PTA), i.e. the widening of the stenosed section of vessel using a balloon catheter, is currently the therapy of choice. However, the disadvantage of this type of therapy is that in 30-50% of the treated patients a restenosis, i.e. a reocclusion, occurs. As an alternative, therefore, surgical treatment is performed by fitting a bypass. However, even with this therapy, restenosis of the bypass vessel occurs in about 50% of patients within 3-6 months after the treatment.

These are the reasons for the development of a number of gene therapy methods in which a second catheter is used to administer a vector having gene therapeutic activity locally on the effected section of vessel (see, for example, WO 95/27070).

Various strategies based on modified balloon catheters have been developed for local administration of the therapeutic gene and are intended to allow direct administration of a substance or the gene into the vessel wall. After local administration with a double-balloon catheter, for example Nabel, E. R. et al. (1990) Science 249, 1285, were able to detect transient expression of the ß-galactosidase gene in transfected cells of the pig femoral artery through liposomal and retroviral transfection. However, they were able to achieve long-lasting expression (up to 5 months) only after retroviral transfection, whereas expression was detectable in liposomally transfected cells for only up to 4 weeks. Besides the duration of transfection, in particular the low transfection efficiency is a limiting factor on liposomal transfer. It was possible to increase the transfection efficiency for example by using HVJ liposomes (Morishita, R. et al. (1994) Gene 149, 13). In this system, inactivated Sendai viruses (hemagglutinating virus of Japan, HVJ) are fused with DNA-containing liposomes. It is possible because of the viral fusion and binding proteins (F and HN protein) for the genetic material to be efficiently inserted directly into the cytoplasm of the cell, bypassing the lysosomes. Finally, it is possible to couple cell-specific antibodies to liposomal (Vingerhoeds, H. et al. (1994) Immunmeth. 4, 259) or viral (Wickham, T. J. et al. (1996) J. Virol. 70, 6831) vectors in order to be able to insert the therapeutic gene, which is under the control of a strong viral promoter, specifically only into the desired cell type.

However, the disadvantages of these methods are that, on the one hand, an additional intervention is necessary and, on the other hand, the efficiency of the local gene transfer in the body is generally not very high. Finally, not all vessels are reached by a catheter, especially not the small-caliber arteries and capillaries. This is important since arteriosclerosis wall lesions not only affect the large vessels but also occur in smaller arteries.

Optimization of tissue-specific expression is of great medical importance in particular for the therapy of vascular disorders. As a result there are possibilities for the therapy not only of direct vascular disorders such as, for example, arteriosclerosis and its sequela (stenosis, restenosis, myocardial infarction), but also all organs to be reached via the bloodstream, and tumors. It is possible eventually via tissue-directed gene transfer into vascular cells for there also to be expression of therapeutic proteins which, because of pathological or genetic alterations in the target organism, are absent or no longer present to a sufficient extent, for example factor VIII deficiency, insulin deficiency etc.

An essential aim of somatic gene therapy is therefore to insert a therapeutic gene after systemic or local administration as specifically as possible into the target cells of the body and to express the therapeutic gene essentially only in these cells. For this purpose, the therapeutic gene on the one hand should be packaged so that it undergoes essentially no degradation by nucleases during transport through the system of blood vessels, and on the other hand it should be in a form which makes cell-specific gene expression possible in the target cells. The specific recognition of cells is also referred to as "targeting". Two possibilities are available in principle for this targeting: On the one hand, antibodies against receptors on the cell surface, which are integrated into viral or liposomal vector systems, can be used (Vingerhoeds, H. et al (1994) supra; Wickham, T. J. (1996) supra), and on the other hand peptides with high binding affinity for receptors on the cell surface can be used.

It has been possible for example with particular antibodies for particular tumor cells to be recognized and detected by means of a suitable label even in the intact tissue structure. However, an essential disadvantage of antibodies is that, for technical reasons, they are usually produced in mice and often lead to serious side effects due to an immune response in patients. Even so-called humanized antibodies are not free of this problem. In a few cases there may be such pronounced immune complexes which drastically impair the blood supply to the kidneys and thus lead to renal failure with a fatal outcome.

It has been possible for example with peptide-presenting libraries to study interactions of proteins with other macromolecules such as sugars, antibodies, lipids or proteins in vitro. Peptide-presenting libraries prepared chemically have been described, for example, by Geysen, H. M. et al. (1986) Mol. Immunol. 23, 709 or de Koster, H. S. (1995) J. Immunol. Methods 187, 179-188. In the peptide synthesis there was use of amino acid mixtures on the solid phase, so that the amino acid sequence of the resulting peptides was statistically, i.e. randomly, distributed. It was also possible in this case to use the peptide libraries in resin-bound form (de Koster, H. S. et al. (1995) supra).

It has been possible with the use of peptide-presenting libraries which are based, for example, on the presentation of the peptides on the surface of phages, to present a large number of different peptides (up to about $10^{11}$ peptides) simultaneously. This entails the peptides being presented for example on the N terminus of the pIII coat protein of filamentous phages (see, for example, Kay, B. K. et al. (1993) Gene 128, 59 or Jellis, C. L. et al. (1993) Gene 137, 63-68). The length of the presented peptides varied from 6 to 38 amino acids. The phage libraries have been used inter alia for epitope screening of antibodies (Cortese, R. et al. (1994) Trends Biotechnol. 12, 262; Grihalde, N. D. et al. (1995) Gene 166, 187), for characterizing protein- or peptide-binding domains (Adey, N. B. & Kay, B. K. (1996) Gene 169, 133; Blond-Elguindi, S. et al. (1993) Cell 75, 717-728; Balass, M. et al. (1993) Proc. Natl. Acad. Sci. USA 90, 10638-10642), for discovering DNA-bindings motifs (Wang, B. et al. (1995) J. Biol. Chem. 270, 23239), for identifying unknown receptors which bind, for example, to viral binding proteins (Hong, S. S. & Boulanger, P. (1995) EMBO J. 14, 4714), for identifying protein-binding peptides (Daniels, D. A. & Lane, D. P. (1994) J. Mol. Biol. 243, 639-652; Hong, S. S. & Boulanger, P. (1995) EMBO J. 14, 4714-4727, No. 19) or antigen-binding single-chain Fv(scFv) fragments (Vaughan, T. J. et al. (1996) Nature Biotechnology 14, 309-314) and have been proposed for targeting cells (Barry, M. A. et al. (1996) Nature Medicine 2, 299-305, No. 3).

Not only peptide-presenting phage libraries but also peptide-presenting bacterial libraries are known. One example thereof comprises fusions of a combinatorial

peptide library with thioredoxin, which led to the identification of peptide aptamers which recognize cyclin-dependent kinase 2 (Colas, P. et al. (1996) Nature, 380, 548-550). The screening of a combinatorial peptide library has been carried out for example also with fusions to the C terminus of the alpha subunit of $G_t$ (340-350) for identifying rhodopsin-binding sequences (Martin, E. L. et al. (1996) J. Biol. Chem. 271, 361-366, No. 1). Another example comprises peptide insertions in a thioredoxin-flagellin fusion protein which is presented on the surface of E. coli cells, with which it is possible to investigate protein-protein interactions (Lu, Z. et al. (1995) Bio/Technology 13, 366-372; U.S. Pat. No. 5,635,182).

The applications described above of the peptide-presenting libraries have been restricted, however, to in vitro investigations with isolated and purified components (sugars, proteins, antibodies).

It was therefore an object of the present invention to find a means for making it possible for diseased organs to be diagnosed and treated in a manner which is targeted and as mild as possible.

One aspect of the present invention is therefore a tissue-binding peptide selected from a peptide having the amino acid sequence

GEGRTVVLSF, AWCRGGILGDAM, GNLVDLVVGFDD, RVSPPKKSGGGV, GSSKWGLTXKCG, RGGVRQRSRGRR, GEGRTVVCRS or SQRWTALWQWIG and variants thereof.

Variants mean according to the present invention deletions, additions or substitutions of one or more amino acids, with a negligible change in the tissue-specific binding of the peptide, i.e. the peptide has retained its ability to bind better to a particular tissue than to another one.

In the case of a deletion, preferably not more than 4 amino acids, especially not more than 2 amino acids, are deleted. The deletion preferably takes place at the N or C

terminus of the peptide, in particular at the N terminus.

The term addition means according to the present invention not only the addition of one or more amino acids, for example 1-25 amino acids, but also fusion proteins of the peptide of the invention with another C- and/or N-terminal peptide or protein. For example, a fusion of a tissue-specific peptide with a peptide which contains a plurality of histidines, for example the peptide GLFHAIAHFIHGGWHGLIHGWYG, leads not only to tissue-specific binding but also to permeabilization of the cell membrane at slightly acidic pH (see, for example, Midoux, P. et al. (1998) Bioconjugate Chem. 9, 260-267). If a fusion protein consists for example of the peptide of the invention and a plurality of lysines, for example 16 lysines in sequence, or the nucleocapsid protein (NCp) 7 of HIV-1 or NCp7-derived peptides, it is possible and particularly simple to complex a nucleic acid, for example a DNA comprising a therapeutic gene, onto the peptide of the invention (see, for example, Harbottle, R. P. et al. (1998) Human Gene Therapy 9, 1037-1047 or Bachmann, A. S. et al. (1998) J. Mol. Med. 76, 126-132). It is thus possible according to the present invention for example also to prepare a fusion protein which comprises a tissue-specific peptide, a peptide which acts to permeabilize the cell membrane, and a nucleic acid-binding peptide, which is particularly advantageous.

In the case of substitutions, preferably conservative substitutions are meant, i.e. substitutions in which for example one or more amino acids with a hydrophobic side chain are replaced by one or more other amino acids with another hydrophobic side chain. Examples of amino acids with hydrophobic side chains are glycine, valine, leucine, isoleucine, methionine, phenylalanine, tryptophan or proline. It is likewise possible for amino acids with a polar neutral residue to be mutually exchanged. Examples thereof are serine, threonine, cysteine, tyrosine, asparagine or glutamine. It is also possible for amino acids with a polar acidic residue to be mutually exchanged. These include, for example, aspartic acid or glutamic acid. Amino acids with a polar basic residue can also be mutually exchanged, such as, for example, lysine, arginine or histidine. There is preferably substitution of not more than up to 4 amino acids, in particular not more than up to 2 amino acids, especially not more than one amino acid.

The term amino acid means according to the present invention not only the amino acids naturally occurring in proteins but also other amino acids which can be incorporated into a peptide chain, such as, for example, hydroxylysine, hydroxyproline, ornithine, citrulline or homocysteine.

The individual amino acids in the peptide of the invention can also be modified. For example, the peptide can be labeled and/or protected by a modification. A suitable label is, for example, replacement of the hydroxyl group of threonine or serine by the isotope $^{18}F$, which has a half-life of 2 hours and can therefore be used satisfactorily for clinical purposes. Alternatively, the peptide can also be labeled with $^{18}F$ by a fluorethylamide linkage at the carboxyl terminus. The C-terminal fluoramidation takes place, for example, with fluorethylamine and a urea derivative (for example TBU) for activation.

Another suitable label is the sulfur isotope $^{35}S$ as label for cysteine or methionine. The isotope $^{35}S$ has the advantage over the isotope $^{18}F$ that it has a longer half-life.

The peptide of the invention can also be labeled with other compounds, for example with ß-galactosidase, or biotin or avidin/streptavidin. A radioactive label particularly with short-lived isotopes has, however, the advantage that the structure of the peptide remains essentially unchanged and the distribution of the peptide in the body can advantageously be analyzed for example with the aid of positron emission tomography (PET).

It is generally advantageous for a label if the functional side chains of the amino acids are previously protected. For this purpose for example the peptide is synthesized on a solid phase using an acid-labile resin (for example SASRIN, a 2-methoxy-4-alkoxybenzyl alcohol-resin, Fréchet, J.M.J. et al. (1979) Polymer 20, 675) from the C to the N terminus by means of the FastMoc® method (Merrifield, R.B. (1963) J. Am. Chem. Soc. 95, 1328; Wang, S.S. (1973) J. Am. Chem. Soc. 95, 1328; Perkin-Elmer Applied Biosystems, Foster City, CA). In this way it is possible for the peptide to be

cleaved off the resin under conditions with which both the side chain protective groups and the N-terminal protective group (Fmoc, N-fluorenylmethoxycarbonyl) remain stable. The label, for example $^{18}F$ can then be introduced. This is generally followed by elimination of the protective groups and purification by HPLC.

Another aspect of the present invention is a nucleic acid coding for a tissue-binding peptide of the invention. A particularly preferred nucleic acid is selected from a nucleic acid having the nucleotide sequence:

GGCGAGGGGCGAACAGTCGTATTGTCGTTCG,
GCCTGGTGTCGGGGGGGTATCCTGGGCGACGCTATG,
GGAAACCTGGTGGATCTAGTTGTGGGTTTTGACGAC,
CGGGTGAGTCCGCCAAAGAAGTCGGGGGGCGGCGTG,
GGGAGTAGCAAGTGGGGATTGACTTAAAAATGTGGG,
CGCGGGGGAGTCCGCCAAAGAAGTCGGGGGCGGCGT,
GGCGAGGGGCGAACAGTCGTATGTCGTTCG or
TCCCAGAGGTGGACTGCACTCTGGCAATGGATCGGG
and variants thereof.

The term variants means according to the present invention in particular variants which, because of the degeneracy of the genetic code, code for the same peptide. The term variants also means, however, the ribonucleic acids (RNA) corresponding to the listed deoxyribonucleic acids (DNA). The DNA or RNA can also be modified in order, for example, to be better protected from degradation by nucleases. Suitable modifications are to be found, for example, in Uhlmann, E. & Peyman A. (1990) Chemical Reviews 90, 543-584 No. 4.

Another embodiment of the present invention relates to a vector comprising one or more nucleic acids of the invention. A vector may be, for example, an expression vector for preparing the peptides of the invention in prokaryotic or eukaryotic cells. Examples of prokaryotic expression vectors are for expression in E. coli for example the vectors pGEM or pUC derivatives and of eukaryotic expression vectors for expression in Saccharomyces cerevisiae for example the vectors p426Met25 or

p426GAL1, for expression in insect cells for example baculovirus vectors as disclosed in EP-B1-0 127 839 or EP-B1-0 549 721, and for expression in mammalian cells for example the vectors Rc/CMV and Rc/RSV or SV40 vectors, all of which are generally available.

In general, the expression vectors also comprise promoters suitable for the particular host cell, such as, for example, the trp promoter for expression in E. coli (see, for example, EP-B1-0 154 133), the ADH2 promoter for expression in yeasts (Russel et al. (1983), J. Biol. Chem. 258, 2674-2682), the baculovirus polyhedrin promoter for expression in insect cells (see, for example, EP-B1-0 127 839) or the early SV40 promoter or LTR promoters for example of MMTV (mouse mammary tumor virus; Lee et al. (1981) Nature 214, 228-232).

Examples of vectors with gene therapeutic activity are virus vectors, preferably adenovirus vectors, in particular replication-deficient adenovirus vectors, or adeno-associated virus vectors, for example an adeno-associated virus vector which consists exclusively of two inverted terminal repeat (ITR) sequences.

For example, in adenoviral vectors, in particular of type 5 (for sequence, see Chroboczek, J. et al. (1992) Virol. 186, 280 - 285) and especially of subgroups C, in general the E1 gene region is replaced by a foreign gene with its own promoter or by the nucleic acid of the invention. The replacement of the E1 gene region, which is a prerequisite for expression of the downstream adenoviral genes, results in an adenovirus incapable of replication. These viruses are then able to replicate only in a cell line which replaces the missing E1 genes.

Replication-deficient adenoviruses are therefore generally formed by homologous recombination in the so-called 293 cell line (human embryonic kidney cell line) which has a copy of the E1 region stably integrated into the genome. For this purpose, the nucleic acid of the invention is cloned into recombinant adenoviral plasmids under the control of a suitable promoter. This is followed by homologous recombination with an E1-deficient adenoviral genome such as, for example, d1327 or del1324 (adenovirus

5) in the 293 helper cell line. Where recombination is successful, viral plaques are harvested. The replication-deficient viruses produced in this way are employed in high titers (for example $10^9$ to $10^{11}$ plaque forming units) for infecting the cell culture or for somatic gene therapy.

The exact site of insertion of the nucleic acid of the invention into the adenoviral genome is not in general critical. It is, for example, also possible to clone the nucleic acid of the invention in place of the deleted E3 gene (Karlsson, S. et al. EMBO J. 5, 1986, 2377 - 2385). However, it is preferred for the E1 region or parts thereof, for example the E1A or E1B region (see, for example, WO 95/00655), to be replaced by the nucleic acid of the invention, especially when the E3 region is also deleted. Also suitable are third-generation adenoviral vectors, i.e. vectors which, apart from the ITR and the packaging signal, comprise no further sequences coding for viral proteins (Kochanek, S. (1996) Proc. Natl. Acad. Sci. USA 93, 5731). Recombination in the helper cell line takes place, for example, with the aid of a helper plasmid.

However, the present invention is not confined to the adenoviral vector system; on the contrary, adeno-associated virus vectors are also particularly suitable in combination with the nucleic acid of the invention because transfer of the nucleic acid of the invention into resting, differentiated cells can be effected by AAV, which is particularly advantageous for the treatment of vascular disorders. Generally sufficient for the vector functions are the two inverted terminal repeat (ITR; see, for example, WO 95/23867) sequences which are about 14 Stp in length. They carry the signals necessary in cis for replication, packaging and integration into the host cell genome. The ability to integrate ensures long-lasting gene expression in vivo, which again is particularly advantageous. A further advantage of AAV is that the virus is non-pathogenic for humans and is relatively stable in vivo. Cloning of the nucleic acid of the invention into the AAV vector or parts thereof takes places by methods known to a skilled worker, as described, for example, in WO 95/23867, in Chiorini, J.A. et al. (1995), Human Gene Therapy 6, 1531 - 1541 or Kotin, R.M. (1994), Human Gene Therapy 5, 793 - 801.

Vectors with gene therapeutic activity can also be obtained by complexing the nucleic acid of the invention with liposomes (neutral or cationic), i.e. the nucleic acid is essentially enclosed in the liposome, because it is possible therewith to achieve a very high transfection efficiency, in particular of myocardial cells (see, for example, WO 95/27070), and the nucleic acid is essentially protected from DNases. Transfection with nucleic acid/liposome complexes using Sendai viruses in the form of so-called HVJ liposomes (virosomes) is particularly advantageous because this makes it possible to increase the transfection rate even further.

In lipofection, small unilamellar vesicles of cationic lipids are prepared by ultrasound treatment of the liposome suspension. The nucleic acid is bound ionically to the surface of the liposomes, specifically in a ratio such that a positive net charge remains and the nucleic acid is 100% complexed by the liposomes. Besides the lipid mixtures DOTMA (1,2-dioleyloxypropyl-3-trimethylammonium bromide) and DOPE (dioleoylphosphatidylethanolamine) employed by Felgner et al. (Felgner, P. L. et al. (1987) Proc. Natl. Acad. Sci USA 84, 7413-7414), numerous new lipid formulations have now been synthesized and tested for their efficiency in transfecting various cell lines (Behr, J.P. et al. (1989), Proc. Natl. Acad. Sci. USA 86, 6982 - 6986; Felgner, J.H. et al. (1994) J. Biol. Chem. 269, 2550 - 2561; Gao, X. & Huang, L. (1991), Biochim. Biophys. Acta 1189, 195 - 203). Examples of the novel lipid formulations are DOTAP N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium ethyl sulfate or DOGS (TRANSFECTAM; dioctadecylamidoglycylspermine). One example of the preparation of DNA-liposome complexes from phosphatidylcholine, phosphatidylserine and cholesterol and the successful use thereof in transfection of the vessel walls with the aid of Sendai viruses is described in WO 95/27070.

It is particularly advantageous if the nucleic acid/liposome complex comprises nucleic acid binding proteins, for example chromosomal proteins, preferably HMG proteins (high mobility group proteins), especially HMG-1 or HMG-2, or nucleosomal histones such as H2A, H2B, H3 or H4, because this makes it possible to increase the expression of the desired nucleic acid by at least 3-10-fold. The chromosomal proteins can be isolated for example from calf thymus or rat liver by generally known methods

or be prepared by genetic manipulation. Human HMG-1 can be prepared, for example, by methods known to the skilled worker particularly simply by genetic manipulation using the human cDNA sequence from Wen, L. et al. (1989) Nucleic Acids Res., 17(3), 1197-1214.

A further aspect of the present invention is therefore also a method for preparing a peptide of the invention, in which the peptide is either chemically synthesized or, as already explained above, prepared by genetic manipulation. The chemical synthesis takes place for example with the aid of the generally known Merrifield technique.

Tissue-binding peptides can, however, also be obtained according to the present invention by bringing one or more peptides into contact with a tissue and then isolating the bound peptide(s).

A further aspect of the present invention is therefore a method for finding a tissue-binding peptide comprising the following steps:

(a) bringing a tissue into contact with one or more peptides, and
(b) isolating one or more tissue-binding peptides.

After a tissue has been brought into contact with one or more peptides in step (a), it is preferred for unbound peptides to be removed for example by washing the tissue with a suitable buffer solution.

Peptide means according to the present invention preferably peptides with a length of about 5 to about 40 amino acids, in particular with a length of about 5 to about 20 amino acids, especially with a length of about 10 to about 20, particularly preferably with a length of about 10 to about 15, amino acids.

Tissue means according to the present invention assemblages of cells, in particular assemblages of cells including their intercellular substance, for example epithelial tissue, connective tissue, supporting tissue, muscle tissue or nerve tissue. The term

tissue also includes according to the present invention whole organs or parts thereof, and vessels. The organs or vessels are preferably still "living" organs or vessels.

The intercellular substance is a substance deposited in the spaces between cells and comprising an apparently structureless ground substance and fibrous connective tissue fibers. It was therefore completely surprising to find, by bringing tissue into contact with one or more peptides, tissue-binding peptides which were essentially specific for the tissue used and were not bound mainly to nonspecific structures such as, for example, the intercellular substance.

The specificity of the tissue-binding peptides can preferably be increased by bringing the peptides isolated in step (b) into contact repeatedly with the tissue one or more times. The peptides are preferably brought into contact with the appropriate tissue up to 10 times after their isolation in each case. It is also possible in this way to find cross-specific peptides which are specific for a plurality of types of tissue, if the peptides are brought into contact successively with different tissues. If, for example, the peptides are brought into contact in portions both with healthy and with diseased tissue, it is possible by a subtractive comparison to find the peptides which are specific for healthy and diseased tissue respectively. Peptides essentially specific only for diseased tissue are suitable, for example, particularly advantageously for diagnosis, for example in the framework of a PET investigation.

It is also known that tissues can undergo pathological changes due to external circumstances such as, for example, stress or inflammations. Thus, for example, smooth muscle cells change their entire phenotype in restenotic vessel areas and are converted from the resting, secretory into the proliferative phenotype. It is consequently possible with the aid of the present invention also to find tissue-binding peptides able to recognize specifically pathologically altered tissue under in vivo conditions. It is also possible in an advantageous manner for pathological cell types in the tissue structure, which have not to date been adequately characterizable, to be characterized, identified and, for example, treated by gene therapy specifically by use of the peptides selected by the method of the invention.

For finding tissue-binding peptides by the method of the invention it is advantageous to use a peptide library, for example a combinatorial peptide library (see, for example, Colas, P. et al. (1996) supra). A peptide library according to the present invention means a collection of a plurality of different peptides which are present in free or in bound form.

A peptide library composed of free peptides comprises, for example, a collection of chemically synthesized peptides (see, for example, Geysen, H. M. et al. (1986) supra). In the peptide synthesis there was use of amino acid mixtures on the solid phase, so that the amino acid sequence of the resulting peptides was distributed statistically. It was possible in this case also to use the peptide libraries in resin-bound form (de Koster, H. S. et al. (1995) supra).

Peptides in bound form are, for example, also so-called peptide-presenting libraries, for example a peptide-presenting phage library or, preferably, a peptide-presenting bacterial library, as already described in detail above. In the peptide-presenting libraries, the peptides are presented in the form of a fusion protein on the surface of, for example, phages or bacteria. Preferred fusions are insertions, preferably fusions with thioredoxin, with thioredoxin(TrxA)-flagellin(FliC), with the alpha subunit of $G_t$, as already described in detail above, or, for example, with LamB (Charbit, A. et al. (1988) Gene 70, 181-189) or Lpp-OmpA (Francisco, J. A. et al. (1993) Proc. Natl. Acad. Sci. USA 90, 10444-10448).

For the present invention, peptide-presenting bacterial libraries and, in particular, those with a thioredoxin fusion protein are particularly preferred especially for selection in an in vivo-approximated perfusion model of isolated vessels and organs, for the following reasons:

1. The peptides are presented as fusion proteins together with a protein, for example thioredoxin, whose three-dimensional structure is generally known. The randomized peptides are generally presented in a very accessible domain located on

the outer surface of the complete protein, for example the so-called Cys-Cys active site loop of thioredoxin. The fact that the peptide is generally presented at an adequate distance from the bacterium and thus readily accessible generally makes a good interaction possible with the desired binding partner.

Consequently, it can generally be ensured that, for example in the case of viral gene transfer into vascular cells, the peptides can display their effect also in the form of fusion proteins with a protein with therapeutic activity, with a negligible loss in their binding ability. Peptides fused to the terminus of a protein and presented in this way in phage libraries generally lose their binding ability as soon as they are expressed as fusion proteins together with a protein with therapeutic activity, which is disadvantageous for use in gene therapy. A considerable reason for this is that, during presentation, the peptides themselves exhibit a free N or C terminus, which is available for binding. It is for this reason particularly advantageous for the peptides to be presented in the form of insertions in selected surface proteins from a peptide-presenting library.

2. For example E. coli thioredoxin is stable to proteases. Consequently, a protease-resistant protein in the form of a fusion protein with the peptide is particularly preferred for example for selection in the ex vivo perfused organ model, in particular because proteases may be present in the vessels on the cells. Organs in particular, for example isolated perfused livers, but also tumors, release proteases to a large extent, and these might cleave the fusion protein in the case of protease sensitivity.

3. Bacteria are unable, owing to their size, to reach the extravascular space as quickly as phages, and thus for example in a perfused organ, to reach cells which are not accessible for later therapy. In contrast to phages, it is possible to incubate corresponding bacterial libraries at body temperature with negligible falsification of the results by endocytosis. This means that it is essentially possible to maintain in vivo conditions also ex vivo, which is particularly advantageous for example for "living" organs and vessels.

The term "living" means according to the present invention organs and vessels still capable of functioning. For retention of the ability to function over a prolonged period it is therefore advantageous if the organ or the vessel is supplied adequately with oxygen and nutrient substrates. Suitable and preferred for this is a nutrient solution containing $Ca^{2+}$, $Mg^{2+}$ and a protein, for example an albumin such as BSA (bovine serum albumin). It is preferred for the concentration of calcium ions to be 2.5 mM and of magnesium ions to be 1.1 mM. Albumin is preferably present in a concentration of 1% in the nutrient solution. Particularly suitable for the perfusion of isolated vessels and isolated organs is so-called Krebs-Henseleit solution, which has the following composition (data in mM): NaCl 116; KCl 4.6; $MgSO_4$ 1.1; $NaHCO_3$ 24.9; $CaCl_2$ 2.5; $KH_2PO_4$ 1.2; glucose 8.3; pyruvate 2.0 and 1% BSA equilibrated with 95% $O_2$ and 5% $CO_2$ (pH 7.4; 37°) (see, for example, Decking, U. K. M. et al. (1997) Circulation Research 81, 154-164, No. 2)

In a preferred embodiment, for example, a peptide-presenting bacterial library was employed in an in vitro vessel perfusion model after previous induction in vivo on the whole animal (rat) of a restenosis in a vessel by denudation of the endothelium using a balloon catheter. This method has the critical advantages that the changes, occurring as a consequence of pathological vascular processes, in the expression pattern of membrane-associated or membrane-bound proteins are retained, the cell polarity is not changed, and all the cells remain in their natural tissue structure.

The peptides found with the aid of the method of the invention can be modified as already described in detail above. One suitable modification is, for example, a radioactive or nonradioactive label for use in a test system or diagnostic aid.

There are numerous applications for the tissue-specific peptides which can be found by the method of the invention, for example the tissue-specific transfer of substances, in particular of pharmaceutically active compounds, especially for tissue-specific gene transfer. Tissue-specific gene transfer is used, for example, for the diagnosis and/or therapy of vascular and/or organic disorders with the aid, for

example, of viral and/or nonviral vectors, preferably with the aid of liposomes as already described in detail above.

For the tissue-specific gene transfer, the peptide can be coupled physically, chemically or by genetic manipulation to the desired nucleic acid (see above). For example, the peptide can either be expressed together with a therapeutic protein, for example nitric oxide synthase (see, for example, WO95/27070), insulin (see, for example, EP-B1-0 001 929), erythropoietin (see, for example, EP-B1-0 148 605), or coagulation factors such as, for example, factor VIII, interferons, cytokines, hormones, growth factors etc., or be coupled to a nucleic acid which is a therapeutic gene, for example a gene coding for the therapeutic proteins mentioned by way of example or for an antisense nucleic acid for the targeted switching off of expression products. Examples of suitable coupling methods are via lysine residues or nucleocapsid proteins as already explained above in detail (see, for example, Harbottle, R. P. et al. (1998) supra or Bachmann, A. S. et al. (1998) supra). It is particularly advantageous if one or more tissue-binding peptides are bound via a positively charged domain, for example polylysine, to one or more nucleic acids.

Suitable for increasing membrane permeation in the gene transfer is also a peptide which increases membrane permeation, for example a histidine-containing polypeptide (see above). It is preferred to use a so-called polyfection solution containing a nucleic acid with the desired therapeutic gene, a fusion protein composed of tissue-specific peptide and a DNA-binding portion, for example a positively charged domain, and a peptide which increases membrane permeation. A suitable polyfection solution is described, for example, in Midoux, P. (1998) supra. In addition, coupling of the peptide to the liposomes via, for example, an introduced C-terminal cysteine onto an activated lipid component, for example via MPB-PE, maleimidophenylbutyrylphosphatidylethanolamine, (Zuidam, N. J. et al. (1995) Biochim. Biophys. Acta 1240, 101) is possible.

The peptides of the invention can also be employed for altering the tropism of viruses, preferably of adenoviruses, for example for tissue-specific gene transfer. This takes

place, for example, by attaching the peptide via a suitable linker to the C terminus of the knob domain (see, for example, Douglas, J. T. & Curiel, D. T. (1997) Neuromuscular Disorders 7, 284-298) or by replacing the region which is responsible for the binding in the knob domain of the fiber protein. An alternative possibility is also to replace the RGD motif in the hypervariable region of the penton base (for example Ad12 19AA, Ad2.5 82AA) by a cell-specific peptide in analogy to the experiments carried out by Wickham with the FLAG epitope, in which case it is likewise preferably possible to eliminate the natural tropism (see Wickham et al. (1996) J. Virol. 70, 6831). The alteration in the tropism can take place, for example, via a shuttle plasmid which codes for the appropriate construct.

Another possible use of the tissue-binding peptides is the use for diagnosing pathologically altered tissue, especially in a pathologically altered vessel, in cases of inflammation, arteriosclerosis, vessels which supply tumor tissue, and tissue with proliferating smooth muscle cells of the vascular system, and the visualization of various sections of the vascular system such as, for example, small/large vessels, veins, organ-specific endothelium.

Diagnosis in these cases can take place either indirectly via, for example, peptide-recognizing antibodies for example in a generally known ELISA test (see, for example Voller, A. et al. (1976) Bull. World Health Organ., 53, 55-63) or directly through additional labeling of the tissue-binding peptide. The label can in this case be a nonradioactive label, for example via biotin or avidin/streptavidin, or else a radioactive label (see above).

A radioactive label is advantageous in particular for a whole-body investigation of the patient with imaging methods such as, for example, PET (positron emission tomography) or SPECT (single photon emission computed tomography) for diagnosing, for example, inflammations, vascular lesions or tumors. This is done by intravenous administration of the labeled peptide to the patient and analysis of the distribution of the peptide in the body for example by means of "whole-body PET".

A further aspect of the present invention is therefore a pharmaceutical and/or test system for example in the form of a diagnostic aid comprising one or more peptides of the invention or one or more peptides which have been found by the method of the invention, or one or more nucleic acids of the invention, and, where appropriate, suitable excipients and/or additives.

Another aspect of the present invention relates also to a composition for example in the form of a transfection or polyfection system comprising one or more peptides of the invention or one or more peptides which have been found by the method of the invention, or one or more nucleic acids of the invention and another substance, preferably a pharmaceutically active compound as already described above in detail, and, where appropriate, suitable excipients and/or additives.

Examples of suitable excipients and/or additives are generally known protease inhibitors and nuclease inhibitors.

The following figures, tables and examples are intended to describe the invention in detail without restricting it:

FIGURES AND TABLES

Fig. 1 shows the Clowes carotid model. After ligature of the internal and external carotid arteries, a balloon catheter is introduced via the external carotid artery into the common carotid artery and dilated, and the vessel is denuded over a distance of 1-1.5 cm. The catheter is then removed again, the external carotid artery is ligated and the internal carotid artery is reopened.

Fig. 2 shows the selection of the peptides in the rat carotid perfused ex vivo.

Fig. 3 shows the relative binding strength of the peptide P36 to a dilated rat carotid removed on postoperative day 8 compared with the untreated control carotid.

Fig. 4 shows the binding constants of the peptide P36 for primary porcine aortic endothelial cells (PAEC), primary porcine smooth muscle cells (VSMC) and monkey kidney cells (COS). The endothelial cells were stimulated by LPS and TNFα (PAEC stimulated).

Tab. 1 shows the DNA sequences and peptide sequences derived therefrom for the isolated clones.

ABBREVIATIONS

| | |
|---|---|
| BSA | bovine serum albumin |
| DCM | dichloromethane, synonym: methylene chloride |
| DIEA | diisopropylethylamine |
| EDT | ethanedithiol |
| HBTU/HOBt | hydroxybenzotriazole |
| IMC | medium, contains (1 x M9 salts, 0.2% (casamino acids, 0.5% glucose, 1 mM $MgCl_2$) 10 x M9 salts: 0.42 M $Na_2HPO_4$, 0.22 M $KH_2PO_4$, 85 mM NaCl, 187 mM $NH_4Cl$, pH 7.4) |
| NMP | N-methyl-2-pyrrolidone |
| PBS | phosphate buffer |
| SASRIN | "super acid sensitive resin" |
| TFA | trifluoroacetic acid |

EXAMPLES

1. Production of a peptide-presenting bacterial library

A peptide-presenting bacterial library which is commercially available from Invitrogen BV, the Netherlands (FliTrx™ Random Peptide Display Library, Catalog No. K1125-01; Z. Lu, et al. (1995) Biotech. 13, 366) is used. In this bacterial library, the bacteria contain an expression cassette which codes for a flagellin and thioredoxin fusion protein. The flagellin serves to anchor the fusion protein in the bacterial membrane, while the thioredoxin presents in an exposed domain a foreign peptide which is 12 amino acids long and whose coding oligonucleotide, which is 36 base pairs long, was cloned at the appropriate site in the thioredoxin-encoding sequence. The oligonucleotide used for the cloning was synthesized in accordance with the random principle so that the bacterial library represents a total of $10^9$ different peptides. The expression cassette is under the control of the tryptophan promoter, so that expression of the fusion protein can be switched on by addition of tryptophan at the time when a bacterial culture is growing exponentially. Since each bacterium takes up only one plasmid, only one type of peptide with a defined amino acid sequence is expressed and presented on the surface by each bacterium.

2. Setting up the bacterial culture

$10^9$ bacteria were added to 50 ml of IMC medium and incubated at +25°C for 15 hours overnight. The cell count was then determined by measuring the $OD_{600}$, $10^{10}$ cells were incubated with 100 µg/ml ampicillin in 50 ml of IMC medium, and expression of the bacterial library was induced by adding 100 µg/µl tryptophan. The cells were incubated at 25°C for a further 6 hours before they were used for selection.

3. In vitro vessel perfusion model

Induction of a restenosis took place in an established rat restenosis model (see, for example, Clowes, A. W. et al. (1983) Lab. Invest. 49, 327).

As depicted in Fig. 1, for example endothelial cells were removed from the common carotid artery of an anesthetized rat by introducing a balloon catheter (denudation). This operation stimulates vascular smooth muscle cells to proliferate and they alter their phenotype from the resting, secretory smooth muscle cell to the proliferating smooth muscle cell.

On the day when proliferation of smooth muscle cells is most pronounced (postoperative day 10), the vessel segment indicated in Fig. 1 was dissected out and perfused with a saline medium with addition of glucose and oxygen aeration in a special ex vivo perfusion system. After an equilibration time of 10 min, which served to wash out all blood cells and blood constituents, this vessel segment was perfused with recirculation with a total of $10^{10}$ peptide-presenting bacteria in oxygen-aerated saline medium at a flow rate of 0.2 ml/min for 60 min. The nonbinding bacteria were detached by treatment with saline medium, and the specifically binding bacteria were eluted by perfusion under stringent conditions with 0.1 M glycine pH 2.0 for 10 minutes. The eluted bacteria were recultivated and subjected to a further five selection cycles (see Fig. 2) to increase the specificity.

4. Perfused rat carotid experimental protocol

Rats weighing about 500 g were anesthetized by administration of 100 µl of Dormicum per 100 g of body weight and 100 µl of Hypnorm per 100 g of body weight. The carotid artery was then exposed cranially of the bifurcation, the internal carotid artery and all the branches originating therefrom were ligated, and the common carotid was denuded with a 2F balloon catheter under 1 bar from the external carotid about 2 cm along the common carotid. The ligature of the internal carotid artery was reopened postoperatively. On postoperative day 10 the denuded

common carotid artery was exposed and perfused ex vivo with 0.1 ml/min PBS with 0.1 M glucose. During this, 10 ml of the tryptophan-induced bacterial suspension was centrifuged at 1 000 rpm for 3 min, washed in PBS and recentrifuged, and the sediment was suspended in a total volume of 6 ml PBS, 0.1 M glucose, 0.1% BSA, 1 mM $CaCl_2$, 10 mM $MgCl_2$. The carotid was then perfused with recirculation at 0.2 ml/min with this suspension and oxygen aeration for 1 h. The suspension was then washed out by perfusion with 2-3 ml of PBS at 0.2 ml/min before the bound bacteria were eluted by elution with 3 ml of 0.1 M HCl (pH 2.2 adjusted with glycine) and grown in 50 ml of IMC medium, 100 µg/ml ampicillin, at +25°C overnight.

5. Results of the selection experiments

The bacteria obtained in the experiments described above express on their surface peptides which bind specifically to proliferating smooth muscle cells. Bacteria obtained after six selections were then plated out on nutrient medium and cultures were set up starting with 60 individual colonies. The plasmid DNA was isolated from these cultivated bacteria and sequenced. Using this model it was surprisingly possible to detect specifically binding peptides with a method which is extremely close to the in vivo situation (organ culture model).

Sequencing of the DNA of the isolated bacteria resulted in the DNA sequences and peptide sequences derived therefrom (see Table 1). The peptide 36 shows homologies in its sequence to $\alpha_4$ integrin which is expressed on leukocytes and is responsible for adhesion of leukocytes to activated vascular cells. Recognition in this case is mediated by the vascular cellular adhesion molecule VCAM-1. The homology is not located in the region characterized as the binding region of the integrin (RGD motif).

6. Binding studies

The peptide from clone 36 (peptide 36) employed for the binding studies was prepared on the 0.1 mmol scale by Fast-Moc solid-phase synthesis in an Applied

Biosystems completely automatic peptide synthesizer. The peptide was radiolabeled for determination of the binding affinities for the desired cell type.

7. Radiolabeling of a tissue-specific peptide

Since the bacteria employed for selection present the peptides on their surface in the context of a fusion protein (thioredoxin), the amino acid occurring at the N terminus (glycine) was used for the radiolabeling. For this purpose, $^{14}$C-glycine was protected with activated N-fluorenylmethoxycarbonylsuccinimide, and peptide present on the resin and protected in the side chains underwent N-terminal covalent coupling using dicyclohexylcarbodiimide. The peptide was then cleaved off the resin, purified by HPLC and investigated in the perfused vessel model and in cell culture for binding to the target cells.

8. Experimental protocol for synthesis of the radiolabeled $^{14}$CFmoc-glycine

20 µmol of the radiolabeled $^{14}$C-glycine in 5 ml of water (equivalent to 1 mCi; DuPont) were introduced together with 38 mg of glycine (0.5 mmol) and 53 mg of $Na_2CO_3$ (0.5 mmol) into a 25 ml round-bottom flask and, while stirring 165 mg (0.49 mmol) of N-fluorenylmethoxycarbonyl carbonate (Fmoc carbonate) dissolved in 5 ml of acetone were added dropwise. During the addition (60 min) the pH was kept constant by adding 1 M $Na_2CO_3$. The mixture was then left to stand at room temperature overnight, and the aqueous phase was acidified by adding 2 M HCl and was extracted twice with 10 ml of ethyl acetate each time. The combined ethyl acetate phases were washed several times with water until neutral and dried with $MgSO_4$, and the product was precipitated by adding petroleum ether and was filtered off. The product was purified by being dissolved in ethyl acetate and slowly precipitating by adding small amounts of petroleum ether.

| | |
|---|---|
| Yield: | 95 mg (corresponding to 68% of theory) |
| | 0.32 mmol |
| specific activity: | 0.0064 MBq/µmol |

9. Experimental protocol for the synthesis of radiolabeled peptides

The labeled Fmoc-glycine was employed for synthesizing a total of 0.4 mmol of peptide so that each peptide was 75% labeled.

The appropriate amount of the resin/peptide mixture (0.1 mmol) was weighed out and put in a glass frit with ground stopper and tap. 25 mg of the $^{14}$C-Fmoc-glycine (0.075 mmol) were dissolved in 1 ml of NMP and, after addition of 155 µl of 0.45 M HBTU/HOBt and 750 µl of 2 M DIEA in NMP, added to the resin/peptide mixture and incubated at room temperature while shaking gently for one hour. Subsequently, to complete the coupling, 150 mg of Fmoc-glycine (0.5 mmol) were dissolved in 2 ml of NMP and, after addition of 2 ml of 0.45 M HBTU/HOBt and 1 ml of 2 M DIEA, added to the reaction mixture. The mixture was incubated at room temperature for 30 min, the unreacted Fmoc-glycine was removed by filtration, and the mixture was washed four times with 6-10 ml of NMP each time. The protective group was eliminated by incubation with 22% piperidine in NMP while stirring for 30 minutes. The piperidine was removed by filtration, and the resin/peptide mixture was subsequently washed four times each with NMP and DCM.

10. Experimental protocol for elimination of protective groups

To eliminate protective groups, the mixture was cooled in ice, and a solution of 0.75 g of phenol, 0.25 ml of EDT, 0.5 ml of thioanisole, 0.5 ml of water and 10 ml of TFA was added with stirring. After incubation with stirring at room temperature for two hours, the liberated peptide was removed by filtration and precipitated by adding five times the volume of ice-cold diethyl ether. After centrifugation, the peptide present in the sediment was washed with diethyl ether and recentrifuged several times.

The peptides were purified by chromatography using an Äkta purifier 100 on an

RPC resource column (3 ml, Pharmacia) with a 20-minute gradient from water, 0.1% TFA to 95% acetonitrile, 0.07% TFA at a flow rate of 2 ml/min, and then lyophilized.

Yields: 65.6 and 70.8% (depending on peptide)
Specific activity: 0.0165 to 0.0182 MBq/µmol

11. Binding studies

For the binding studies in the perfused vessel model and in cell culture for binding to the target cells, the vessels or the cells were incubated with various concentrations of the peptides to be investigated, for various intervals of time, and the amount of bound peptide per cm of vessel or per number of cells was determined.

11.1 Binding to the perfused carotid

It is evident from Fig. 3 that the binding of peptide 36 in ex vivo perfusion is about four times stronger to the dilated carotid than to the untreated control.

11.2 Binding to porcine endothelial cells in cell culture

Since peptide 36 also shows homologies with the porcine $\alpha_4$ integrin sequence, in addition cell culture investigations were carried out on porcine endothelial cells (PAEC) which have been stimulated by treatment with lipopolysaccharides (LPS) and tumor necrosis factor (TNF$\alpha$). This stimulation corresponds to an in vivo activation of cells which are localized in vessel areas exposed to stress or inflammatory stimuli and are thus also localized in restenotic vessel areas.

In these experiments the peptides were able to bind both specifically to smooth muscle cells (VSMCs) and to activated PAECs. Surprisingly, the peptide in fact binds more strongly to activated PAECs than to VSMCs. In addition, the binding constants of the

peptide for activated and nonactivated PAECs differ by almost 3 powers of ten (Fig. 4). There is virtually no binding of the peptide to control cells (COS).

It will be apparent to those skilled in the art that various modifications and variations can be made to the compositions and processes of this application. Thus, it is intended that the present invention cover such modifications and variations, provided they come within the scope of the appended claims and their equivalents.

Priority application DE 198 45 4934.1, filed on October 2, 1998, including the specification, drawings, claims and abstract, is hereby incorporated by reference. All publications cited herein are incorporated in their entireties by reference.

PCT/EP99/07296

Dr. Jürgen Schrader

December 13, 2000

C 27609PC BÖ/ATE/ps

Patent Claims

1. A tissue-binding peptide selected from a peptide having the amino acid sequence
GEGRTVVLSF, AWCRGGILGDAM, GNLVDLVVGFDD,
RVSPPKKSGGGV, GSSKWGLTXKCG, RGGVRQRSRGRR,
GEGRTVVCRS or SQRWTALWQWIG and variants thereof.

2. A nucleic acid coding for a tissue-binding peptide as claimed in claim 1.

3. A nucleic acid as claimed in claim 2 selected from a nucleic acid having the nucleotide sequence
GGCGAGGGGCGAACAGTCGTATTGTCGTTCG,
GCCTGGTGTCGGGGGGGTATCCTGGGCGACGCTATG,
GGAAACCTGGTGGATCTAGTTGTGGGTTTTGACGAC,
CGGGTGAGTCCGCCAAAGAAGTCGGGGGGCGGCGTG,
GGGAGTAGCAAGTGGGGATTGACTTAAAAATGTGGG,
CGCGGGGGAGTCCGCCAAAGAAGTCGGGGGCGGCGT,
GGCGAGGGGCGAACAGTCGTATGTCGTTCG or
TCCCAGAGGTGGACTGCACTCTGGCAATGGATCGGG
and variants thereof.

4. A vector comprising a nucleic acid as claimed in claim 2 or 3.

5. A method for preparing a tissue-binding peptide as claimed in claim 1, characterized in that the peptide is prepared either by chemical synthesis or by genetic manipulation.

6. A method for finding a tissue-binding peptide where the peptide(s) is (are) present in peptide-presenting bacterial library and the method comprises the

following steps:

(a) bringing a tissue into contact with one or more peptides, and

(b) isolating one or more tissue-binding peptides.

7. The method as claimed in claim 6, characterized in that the peptides isolated in step (b) are brought into contact repeatedly one or more times with the same or with a different tissue.

8. The method as claimed in claim 6 or 7, characterized in that the tissue is a diseased tissue.

9. The use of a tissue-binding peptide as claimed in claim 1 or obtained by the method as claimed in any of claims 6-8 for the tissue-specific transfer of substances, in particular of pharmaceutically active compounds, especially for tissue-specific gene transfer.

10. The use as claimed in claim 9, characterized in that the gene transfer proceeds virally and/or nonvirally, preferably with the aid of liposomes.

11. The use as claimed in claim 9 or 10, characterized in that one or more tissue-binding peptides are bound via a positively charged domain to one or more nucleic acids.

12. The use of a tissue-binding peptide as claimed in claim 1 or obtained by the method as claimed in any of claims 6-8 for altering the tropism of viruses, in particular of adenoviruses.

13. The use of a tissue-binding peptide as claimed in claim 1 or obtained by the method as claimed in any of claims 6-8 for providing a diagnostic aid for visualizing pathologically altered tissue and/or various sections of the vascular system, preferably small/large vessels, veins or organ-specific endothelium.

14. The use as claimed in claim 13, characterized in that the pathologically altered tissue is selected from a pathologically altered vessel; in cases of inflammation; arteriosclerosis; vessels which supply tumor tissue; tissue with proliferating smooth muscle cells of the vascular system.

15. The use as claimed in claim 13 or 14, characterized in that the tissue-binding peptide is labeled, preferably radiolabeled.

16. A pharmaceutical and/or diagnostic aid comprising one or more tissue-binding peptides as claimed in claim 1, one or more nucleic acids as claimed in any of claims 2-4 or one or more tissue-binding peptides obtained by the method as claimed in any of claims 6-8, and, where appropriate, suitable excipients and/or additives.

17. A composition comprising one or more tissue-binding peptides as claimed in claim 1, one or more nucleic acids as claimed in any of claims 2-4 or one or more tissue-binding peptides obtained by the method as claimed in any of claims 6-8, and another substance, preferably a pharmaceutically active compound, and, where appropriate, suitable excipients and/or additives.

**Fig. 1a**

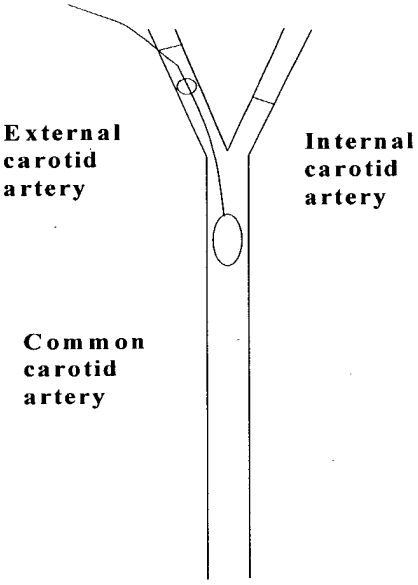

External carotid artery
Internal carotid artery
Common carotid artery

**Fig. 1b**

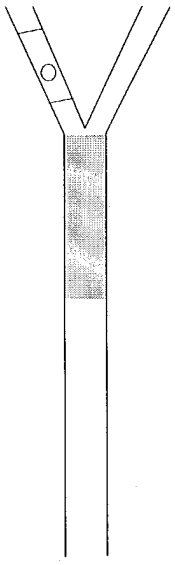


**Fig. 2**

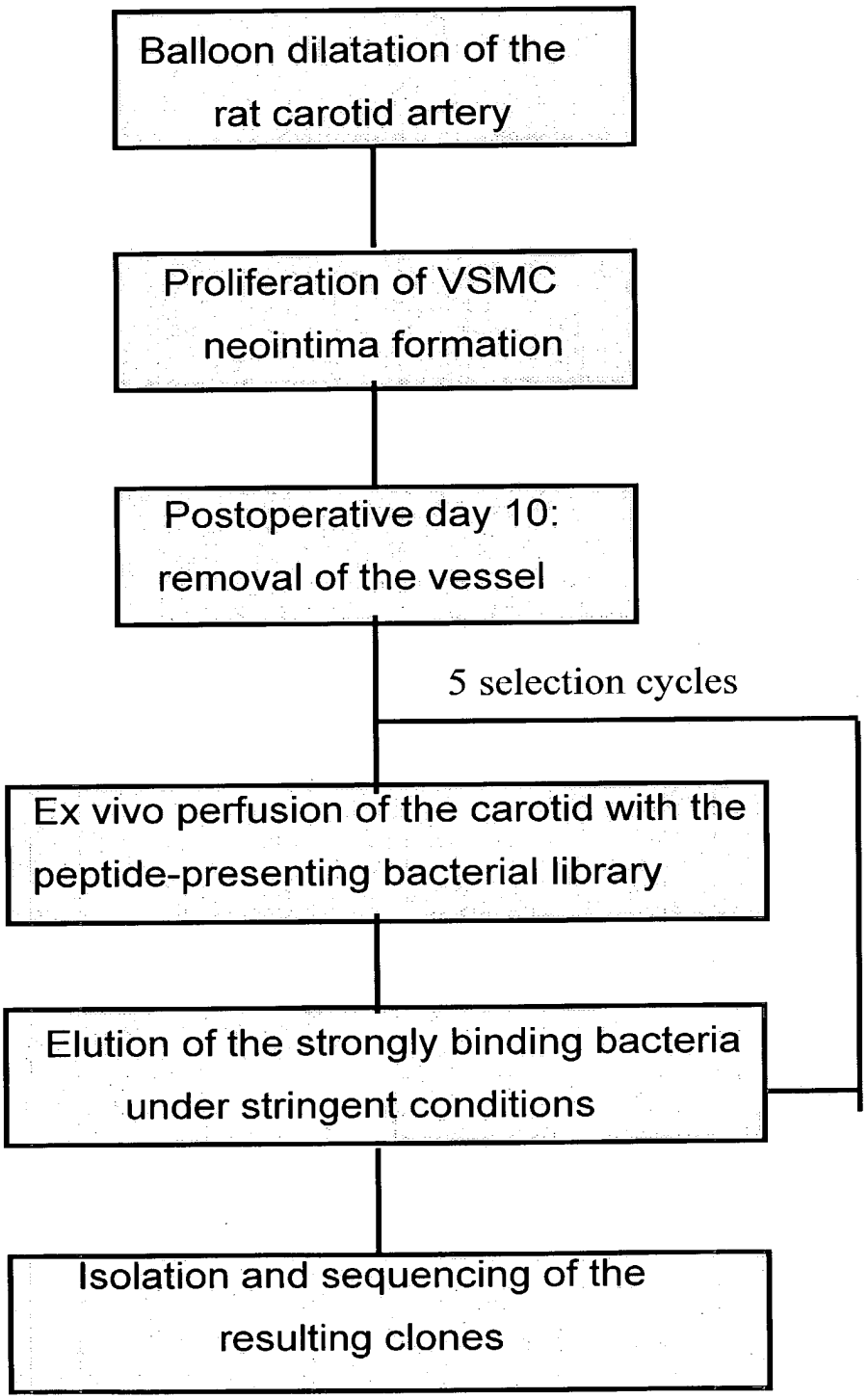

Balloon dilatation of the rat carotid artery
Proliferation of VSMC neointima formation
Postoperative day 10: removal of the vessel
5 selection cycles
Ex vivo perfusion of the carotid with the peptide-presenting bacterial library
Elution of the strongly binding bacteria under stringent conditions
Isolation and sequencing of the resulting clones

**Fig. 3**

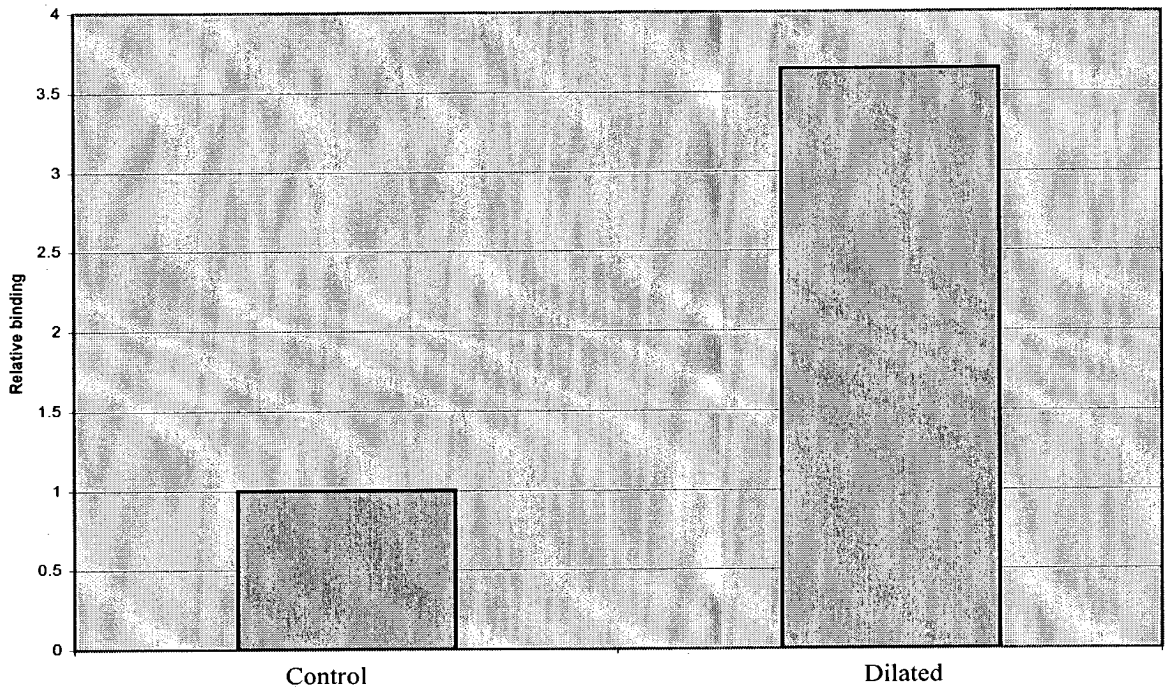
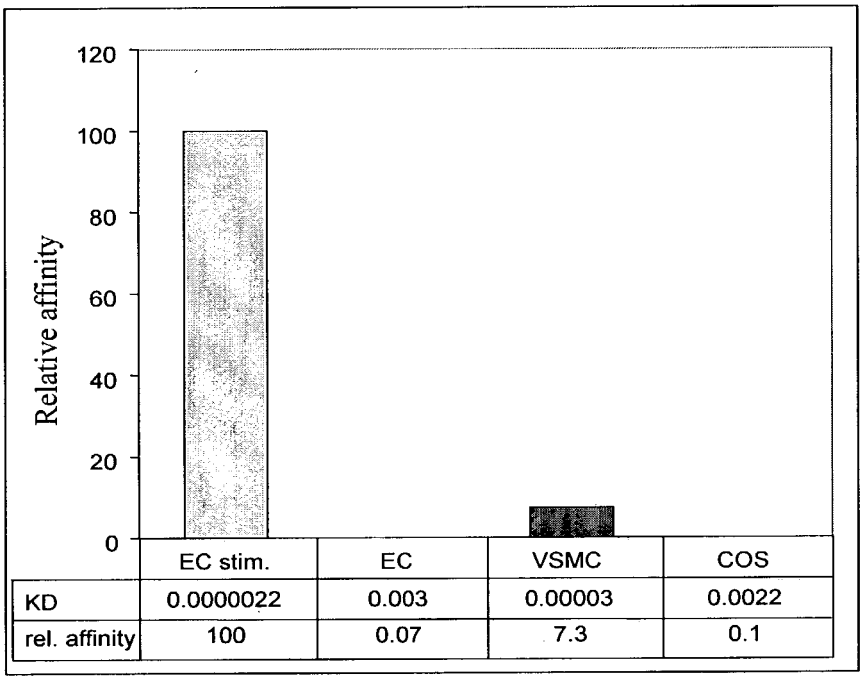


**Fig. 4**




| | EC stim. | EC | VSMC | COS |
|---|---|---|---|---|
| KD | 0.0000022 | 0.003 | 0.00003 | 0.0022 |
| rel. affinity | 100 | 0.07 | 7.3 | 0.1 |

# Tab. 1

| Clone | DNA sequence | AA sequence |
|---|---|---|
| 3 | GGCGAGGGGCGAACAGTCGTATTGTCGTTCG | GEGRTVVLSF |
| 4, 13 | GCCTGGTGTCGGGGGGGTATCCTGGGCGACGCTATG | AWCRGGILGDAM |
| 7, 23 | GGAAACCTGGTGGATCTAGTTGTGGGTTTTGACGAC | GNLVDLVVGFDD |
| 9 | CGGGTGAGTCCGCCAAAGAAGTCGGGGGGCGGCGTG | RVSPPKKSGGGV |
| 17,20,21, 22,29,30 | GGGAGTAGCAAGTGGGGATTGACTTAAAAATGTGGG | GSSKWGLTXKCG |
| 19 | CGCGGGGGAGTCCGCCAAAGAAGTCGGGGGCGGCGT | RGGVRQRSRGRR |
| 32 | GGCGAGGGGCGAACAGTCGTATGTCGTTCG | GEGRTVVCRS |
| 36 | TCCCAGAGGTGGACTGCACTCTGGCAATGGATCGGG | SQRWTALWQWIG |

**PATENT**
**ATTORNEY DOCKET NO: 50125/022001**

## COMBINED DECLARATION AND POWER OF ATTORNEY

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled TISSUE-BINDING PEPTIDES, THEIR IDENTIFICATION, PREPARATION AND USE, the specification of which

- ☐ is attached hereto.
- ■ was filed on March 30, 2001 as Application Serial No. 09/806,555 and was amended on _____.
- ☐ was described and claimed in PCT International Application No. _____ filed on _____ and as amended under PCT Article 19 on _____.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose all information I know to be material to patentability in accordance with Title 37, Code of Federal Regulations, § 1.56.

**FOREIGN PRIORITY RIGHTS**: I hereby claim foreign priority benefits under Title 35, United States Code, § 119 of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed:

| Country | Serial Number | Filing Date | Priority Claimed? |
|---|---|---|---|
| PCT | PCT/EP99/07296 | October 1, 1999 | Yes |
| GERMANY | DE 19845434.1 | October 2, 1998 | Yes |

**PROVISIONAL PRIORITY RIGHTS**: I hereby claim priority benefits under Title 35, United States Code, § 119(e) and § 120 of any United States provisional patent application(s) listed below filed by an inventor or inventors on the same subject matter as the present application and having a filing date before that of the application(s) of which priority is claimed:

## COMBINED DECLARATION AND POWER OF ATTORNEY

| Serial Number | Filing Date | Status |
|---|---|---|
| | | |

**NON-PROVISIONAL PRIORITY RIGHTS**: I hereby claim the benefit under Title 35, United States Code, § 120 of any United States application(s) listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code, § 112, I acknowledge the duty to disclose all information I know to be material to patentability as defined in Title 37, Code of Federal Regulations, § 1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

| Serial Number | Filing Date | Status |
|---|---|---|
| | | |
| | | |

I hereby appoint the following attorneys and/or agents to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith: Paul T. Clark, Reg. No. 30,162, Karen L. Elbing, Ph.D. Reg. No. 35,238, Kristina Bieker-Brady, Ph.D. Reg. No. 39,109, Susan M. Michaud, Ph.D. Reg. No. 42,885, Mary Rose Scozzafava, Ph.D., Reg. No.36,268, James D. DeCamp, Ph.D., Reg. No. 43,580, Sean J. Edman, Reg. No. 42,506, Timothy J. Douros, Reg. No. 41,716.

Address all telephone calls to: Karen L. Elbing, Ph.D. at 617/428-0200.

Address all correspondence to: Karen L. Elbing, Ph.D. at Clark & Elbing LLP, 176 Federal Street, Boston, MA 02110. **Customer No: 21559**

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patents issued thereon.

## COMBINED DECLARATION AND POWER OF ATTORNEY

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|---|---|---|---|
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| Signature: Jürgen Schrader | | | Date: 15 Jun 2001 |

| Full Name (First, Middle, Last) | Residence Address (City, State, Country) | Post Office Address (Street, City, State, Country) | Citizenship |
|---|---|---|---|
| 2 Andreas Herrmann | Köln, Germany DEX | Löwenburgstrasse 26<br>50939 Köln, Germany | German |
| Signature: A. Herrmann | | | Date: 05-29-01 |

SEQUENCE LISTING

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